



# Spectroscopy as a useful tool for the identification of changes with time in post-mortem vitreous humor for forensic toxicology purposes

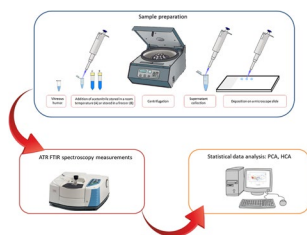
Anna Wójtowicz<sup>1</sup> · Agata Mitura<sup>1</sup> · Renata Wietecha-Posłuszny<sup>1</sup> · Rafał Kurczab<sup>2</sup> · Marcin Zawadzki<sup>3</sup>

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## Abstract

Vitreous humor (VH) is an alternative biological matrix with a great advantage of longer availability for analysis due to the lack of many enzymes. The use of VH in forensic toxicology may have an added benefit, however, this application requires rapid, simple, non-destructive, and relatively portable analytical analysis methods. These requirements may be met by Fourier transform infrared spectroscopy technique (FT-IR) equipped with attenuated total reflection accessory (ATR). FT-IR spectra of vitreous humor samples, deposited on glass slides, were collected and subsequent chemometric data analysis by means of Hierarchical Cluster Analysis and Principal Component Analysis was conducted. Differences between animal and human VH samples and human VH samples stored for diverse periods of time were detected. A kinetic study of changes in the VH composition up to 2 weeks showed the distinction of FT-IR spectra collected on the 1st and 14th day of storage. In addition, data obtained for the most recent human vitreous humor samples—collected 3 and 2 years before the study, presented successful discrimination of all time points studied. The method introduced was unable to detect mephedrone addition to VH in the concentration of 10 µg/cm<sup>3</sup>.

## Graphic abstract



**Keywords** Vitreous humor · Human sample · Post-mortem analysis · Forensic toxicology · FT-IR spectroscopy

✉ Renata Wietecha-Posłuszny  
wietecha@chemia.uj.edu.pl;  
renata.wietecha-posluszny@uj.edu.pl

<sup>1</sup> Laboratory for Forensic Chemistry, Department of Analytical Chemistry, Faculty of Chemistry, Jagiellonian University, Gronostajowa 2, 30-387 Kraków, Poland

<sup>2</sup> Faculty of Mathematical and Natural Sciences, Department of Chemistry, University of Applied Sciences in Tarnów, Mickiewicza 8, 33-100 Tarnów, Poland

<sup>3</sup> Department of Forensic Medicine, Medical University in Wrocław, 4 Jana Mikulicza-Radeckiego St., 50-345 Wrocław, Poland

## Introduction

Vitreous humor (VH) is a gelatinous matrix that fills the space between the lens and the retina. VH consists mainly of water (> 98%) and other components such as collagens (type II, V, VI, IX, and X), glycosaminoglycans (GAG), inorganic salts, and lipids [1–3]. In the VH composition were found also, soluble non-structural proteins such as serum components which are normally present in lower concentrations than in serum [4]. In addition, vitreous humor contains a low number of structural cells—hyalocytes [5]. The exact

composition and viscosity of VH samples depends on the anatomical region where the sample is taken, the state of the lenses and the presence of vitreous pathologies [2]. Furthermore, it was reported that human vitreous humor undergoes aging changes throughout life that include collagen fibrils aggregation [6]. Studies also showed that mammalian vitreous humor differs between species. Human eyes were reported to contain higher concentration of collagens than bovine vitreous [5] and to contain the hCA II as well as hCA IV isoenzymes [7]. Vitreous humor was successfully applied as an alternative matrix in post-mortem forensic toxicology, especially in cases when commonly used matrices, such as blood and urine, were not available due to advanced putrefaction, skeletonisation, exhumation or air accidents [8, 9]. The greatest advantage of VH over classical matrices is that it lacks many enzymes and is well protected against putrefaction and activity of microorganisms, so it can be analyzed for a longer period of time [8].

Numerous studies were performed on the drug determination in the vitreous humor matrix. Quantification of cocaine [10, 11], amphetamines [10, 12], opiates [10, 13], cannabinoids [10, 14], benzodiazepines [15, 16], antidepressants [17, 18], gamma-hydroxybutyric acid (GHB) [19], synthetic opioids [20, 21], synthetic cathinones [22], and their metabolites in vitreous humor was reported. Designer drugs from the group of synthetic cathinones such as mephedrone, which chemical structure is shown in Fig. 1, are popular recreational substances especially among youths, however, their abuse has been associated with toxicity and drug-related deaths have been reported [23]. There is still a lack of data available on human and animal toxicity, potential overdose and long-term effects or the pharmacokinetics and pharmacodynamics of synthetic cathinones [24]. Mephedrone was reported to be extensively metabolized as a substrate of cytochrome P450 2D6, however, still around 15% of the absorbed dose was detected unchanged in urine. Four metabolites of mephedrone: nor-mephedrone, *N*-succinyl-nor-mephedrone, 1'-dihydromephedrone, and 4'-carboxymephedrone have been successfully determined in human plasma and urine and in rat brain homogenates. Mephedrone and nor-mephedrone were the main active compounds found in the brain, and therefore, the compounds with the greatest ability to cross the blood–brain barrier in vivo [25]. However, there is still little information on mephedrone pharmacokinetics in vitreous humor. The utility of VH matrix in the

analysis of mephedrone was reported by Adamowicz et al. [24], when in a case of fatal intoxication, the drug was found in vitreous humor at the concentration of  $7.1 \mu\text{g}/\text{cm}^3$  and in blood at the concentration of  $5.5 \mu\text{g}/\text{cm}^3$ .

Analytical techniques most widely used for successful VH drug determination are separation techniques, including liquid and gas chromatography, coupled to mass spectrometry detectors [26]. The use of these techniques makes analysis a time-consuming, labor-intensive and destructive process. Contemporary trends in drug analysis of vitreous humor were critically evaluated by Wójtowicz et al. [27] in a recent review. For the purposes of forensic toxicology, consideration should be given to the application of methods which do not require complicated sample preparation and prevent sample destruction [27].

Fourier transform infrared (FT-IR) spectroscopy is a vibrational spectroscopy technique that enables fast, non-destructive, and relatively inexpensive analysis. Its multiple applications for the analysis of biological samples have already been reported including screening and diagnosis of diseases such as colitis [28], brain tumor [29], cancer [30, 31], and even depression [32]. Forensically, relevant biological samples have also been analyzed by FT-IR spectroscopy for the identification of blood [33] and blood's aging [34], and hair's identification [35]. In addition, ATR-FT-IR spectroscopy has recently been used to evaluate the effect of cause of death on the estimation of the post-mortem interval from seven rats organs including the brain, heart, lung, liver, spleen, kidney, and skeletal muscle [36]. Non-biological traces such as paints [37] cosmetics [38], fibers [39], gunshot residues [40], and gasoline [41] have also been studied using FT-IR spectroscopy.

All the advantages of FT-IR spectroscopy make it a useful tool in the analysis of vitreous humor samples. Zhang et al. [42] have already proven that vitreous humor can be analyzed by FT-IR spectroscopy. Study on prediction of post-mortem interval in rabbit vitreous humor samples was performed by the group [42]. Absorption bands assigned to amides of peptide backbone, lipids, free fatty acids, free amino acids, polypeptides, other carboxylic acids, carbohydrates, phosphoric acids, DNA, and RNA were observed in the  $1900\text{--}900 \text{ cm}^{-1}$  range of VH spectrum. Table 1 shows the assignment of FT-IR spectral bands recorded for rabbit VH samples in the  $1900\text{--}900 \text{ cm}^{-1}$  range [42]. Zhang et al. found the  $1313$  and  $925 \text{ cm}^{-1}$  absorption bands to be highly correlated with post-mortem intervals. Further study enabled the identification of fatal hypothermia from rabbit VH samples based on changes in their FT-IR spectrum [43].

The aim of this study was to test the possible application of FT-IR spectroscopy in toxicological analysis of post-mortem vitreous humor samples. FT-IR spectra of post-mortem human and animal VH samples were compared and kinetic study was conducted to capture changes occurring in VH

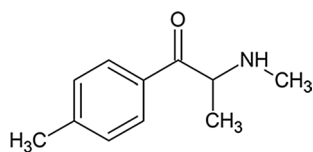


Fig. 1 Chemical structure of mephedrone [23]

**Table 1** Assignment of FT-IR spectral bands from rabbit VH samples

Wavenumber/ cm <sup>-1</sup>	Mode assignment	Compound assignment
1663	Amide I	Proteins
1586	N–H bending and C–N stretching	Proteins
1454	C–H stretching from CH <sub>2</sub> and CH <sub>3</sub>	Lipids
1409	C=O vibration of COO <sup>-</sup>	Free fatty acids, free amino acids, polypeptides, and other carboxylic acids
1313	Amide III	Proteins
1121	C–O, C–OH, and P–O vibration	Carbohydrates, phosphoric acids, DNA, and RNA
1083		
1041		
925		

throughout 2 weeks storage. Two ways of sample deproteization were applied and compared on FT-IR results obtained. Moreover, FT-IR spectra of VH after addition of mephedrone in low concentration (10 µg/cm<sup>3</sup> as similar to the VH level reported in a fatal mephedrone intoxication [24]) were recorded to check if the method is able to detect drugs in this toxicologically relevant concentration. A two-week kinetics study was also performed on mephedrone containing samples to track the possible impact of drugs metabolism on VH spectrum changes with time.

## Results and discussion

### Differentiation between human and animal VH samples

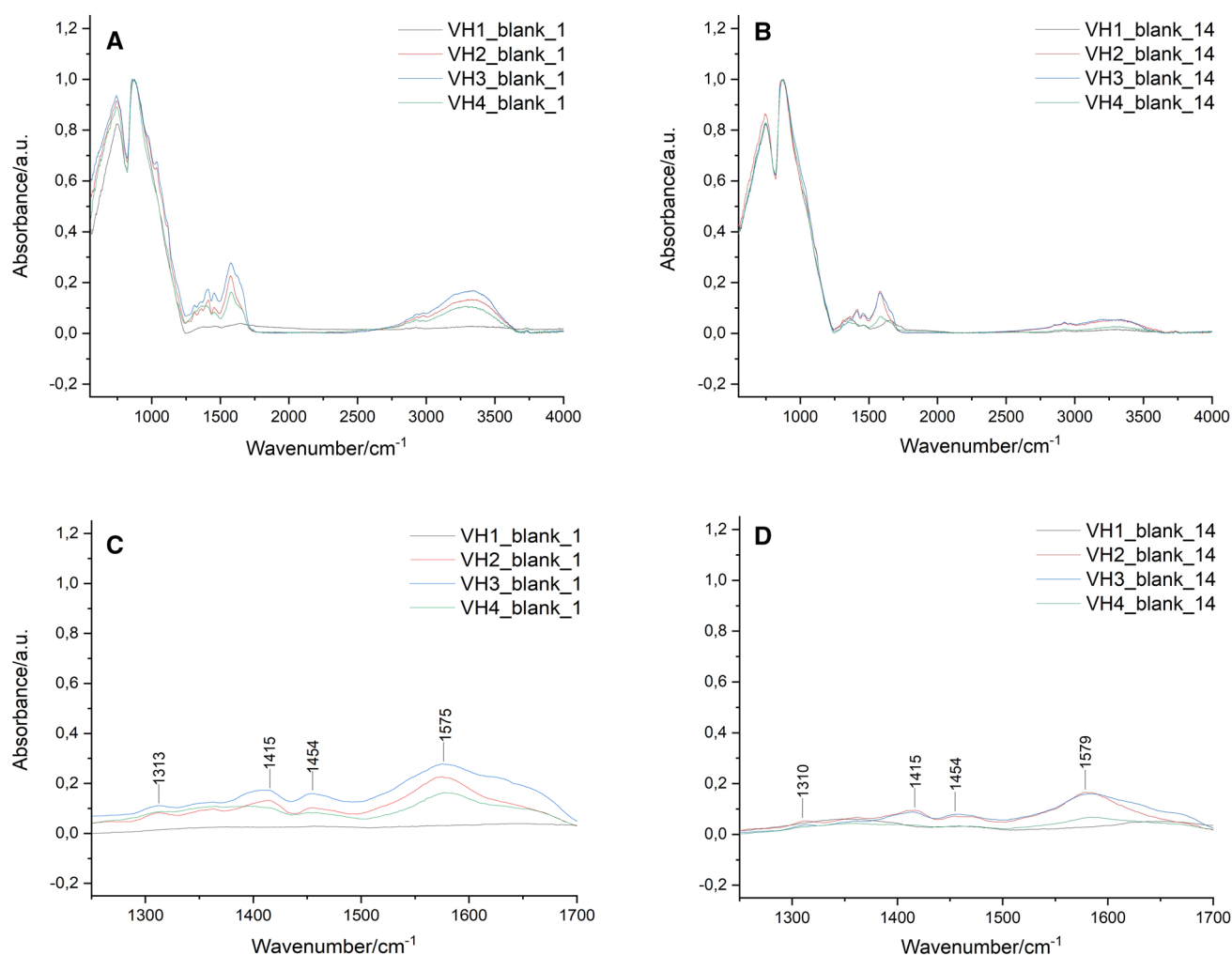
Throughout the entire study, visual comparison of FT-IR spectra obtained for human (VH1, VH2, VH3) and bovine (VH4) vitreous humor samples showed that they differ most significantly in the spectral range between 1250 and 1700 cm<sup>-1</sup>. A comparison of spectra recorded in the full range studied and the range 1250–1700 cm<sup>-1</sup> are presented in Fig. 2. The spectra were obtained after averaging three replicates of spectra obtained for blank samples obtained after deproteinization with ACN stored at room temperature and three replicates of spectra obtained for samples where ACN stored in the freezer was used, as none statistical differences between these two groups were observed and will be further discussed. A strong band appearing below 1250 cm<sup>-1</sup> was assigned to a glass slide substrate, as can be seen on a blank spectrum of the glass slide presented in Fig. 3. The spectrum recorded confirms that the glass substrate provides a large spectral window to detect changes in the sample composition.

The discrimination of VH groups was the greatest on the first days of study and got worse over time. A comparison of VH spectra in the range between 1250 and

1700 cm<sup>-1</sup> obtained on 1st and 14th measuring day is presented in Fig. 2. As can be seen, the spectral characteristic of human VH2 and VH3 samples was the most similar and bands assigned to proteins (~ 1313, 1586 cm<sup>-1</sup>), lipids (~ 1454 cm<sup>-1</sup>), and carboxylic acids (~ 1409 cm<sup>-1</sup>) are visible. However, on the spectrum of the oldest human sample (VH1), the presence of these characteristic bands is not clearly outlined, which impacts its distinction. The same bands are also present on the bovine VH spectrum, however, their relative intensity and position differs. That might be a result of different proteins composition of human and animal vitreous humor [5]. In addition, after 14 days, spectral bands present on the bovine VH sample spectrum lose their intensity and the spectral characteristic of VH4 gets closer to VH1 that worsens these sample distinctions.

The distinction between VH samples studied may be clearly visible when chemometric methods are applied. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were used throughout the study. The analyses were carried in the full spectral range from 550 to 4000 cm<sup>-1</sup>. The comparison of results from the 1st and 14th measuring day is presented in Figs. 4 and 5 for HCA and PCA analyses, respectively. The number of PCs needed to describe the data was selected based on scree plots shown in Fig. 6. PC1 and PC2 accounted for 96% of the variation for the data from the 1st measuring day and 90% for the 14th day. As was mentioned before in the analysis of spectra, the distinction worsens over time. However, even when results from all measuring days are compared there is a significant trend visible in the distinction of VH spectra. Results obtained after PCA analysis of VH samples from all measuring days is presented in Fig. 7.

Clear distinction of both younger human vitreous humor samples and the bovine sample puts into a question the application of animal VH as a surrogate matrix. Differences between these two matrices can be captured by a method as simple as FT-IR. Furthermore, the age of a VH sample is another issue that should be taken into account as significant



**Fig. 2** Comparison of VH spectra in the full spectroscopic range ( $550\text{--}4000\text{ cm}^{-1}$ ) obtained on **A** 1st and **B** 14th measuring day, and in the range between  $1250$  and  $1700\text{ cm}^{-1}$  obtained on **C** 1st and **D** 14th

measuring day for blank human (VH1, VH2, VH3) and bovine (VH4) samples stored in a fridge at  $4\text{ }^{\circ}\text{C}$

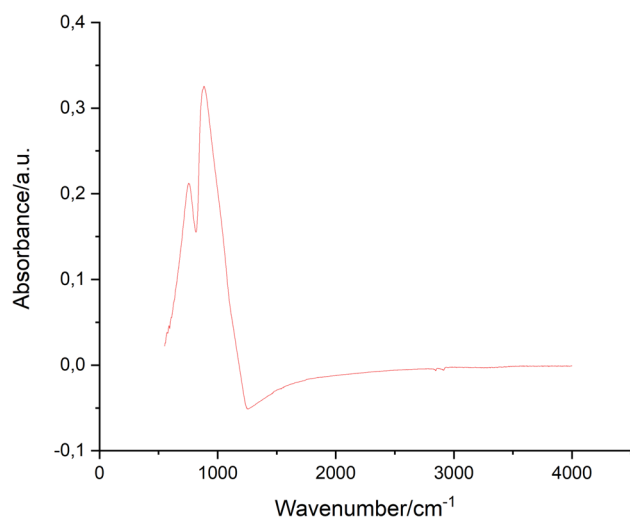
differences can be seen between samples collected over and below 5 years.

### Time changes in the vitreous sample composition

Throughout 2-week study, time changes in the FT-IR spectra of vitreous humor samples were visible. Visual comparison of the spectra shows intensity changes of the band around  $1630\text{ cm}^{-1}$ , which can be assigned to the amide I region. Amide I component usually occurs in the spectral range from  $1600$  to  $1700\text{ cm}^{-1}$  and arises mainly from stretching vibrations of main-chain. Furthermore, Zandomeneghi et al. [44] reported that the characteristic of this band relates to the  $\beta$ -sheet twist and the number of strands per sheet. Figure 8 presents how the intensity of this band was changing for human VH2 sample. As can be seen, changes are non-linear and the lowest intensity is observed for 1st and 14th

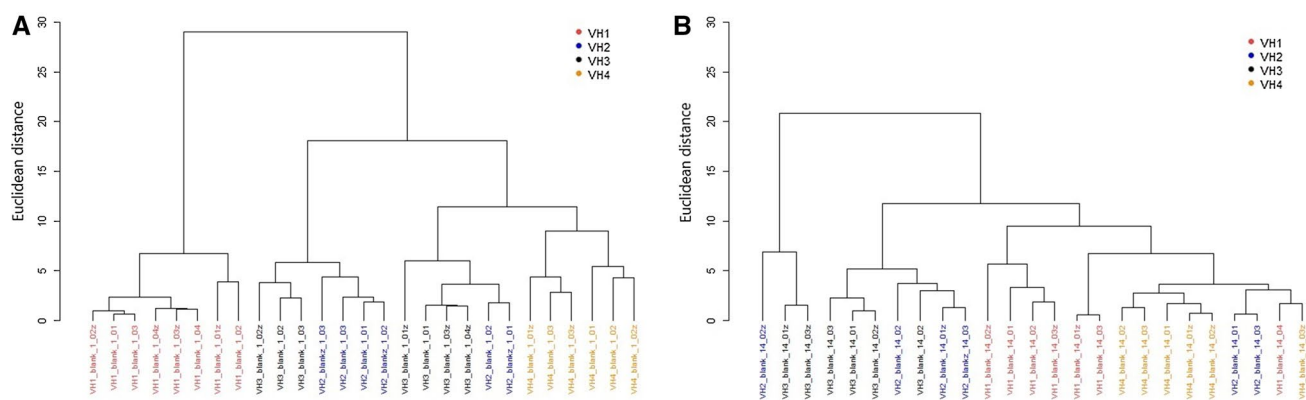
measuring day, while highest intensities are characteristic for days 3–9. These intensity changes may indicate time changes in the structure of VH proteins. However, a thorough explanation of the non-linearity of the changes requires further intensive research. Some explanation can be found in the intricate structure of the amide I band. The deconvolution of this band done by Litvinov et al. [45] illustrates the contribution of individual secondary structure elements, such as  $\beta$ -sheets,  $\alpha$ -helix, random coils, etc. All these elements might behave differently under the influence of changing environmental conditions.

HCA and PCA statistical analyses of the changes within samples were also conducted. PCA results provided better data discrimination so they are included in the study. Figure 9 presents PCA results obtained for time changes in the spectral characteristic of all samples studied. The number of PCs needed to describe the data was selected based on scree

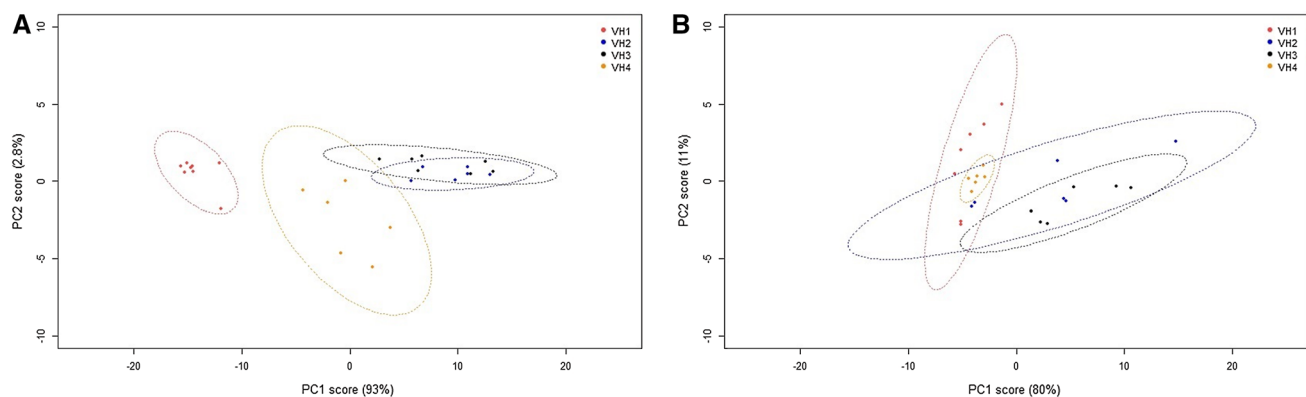


**Fig. 3** FT-IR spectrum of a glass slide purchased from Equimed company

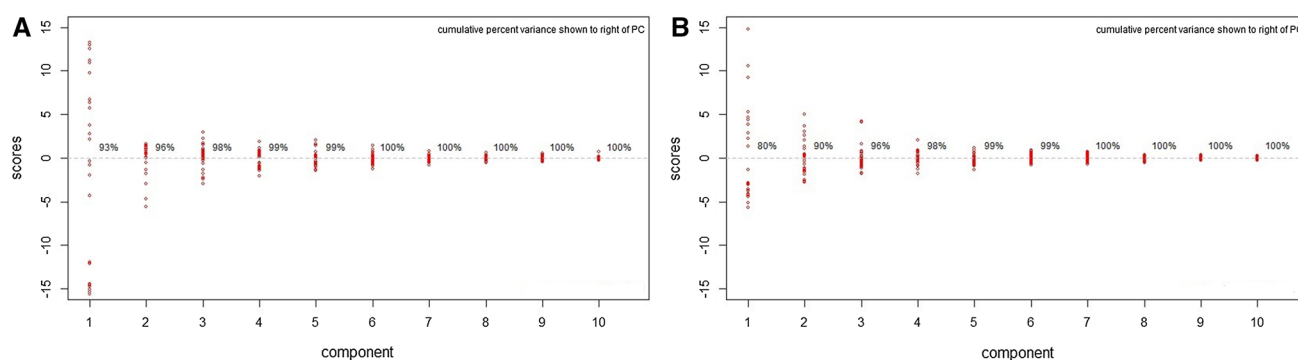
plots shown in Fig. 10. PC1 and PC2 for samples VH2, VH3, and VH4 accounted for 94–97% of the variation. However, for VH1, three PCs were needed to explain 90% of the variation. For comparison purposes, in Fig. 9, the PCA results for the first two components are presented. As shown in Fig. 9, for human vitreous samples VH2 and VH3, appropriate measuring days discrimination is visible along PC1. The PC1 score increases linearly from 1st to 9th day with 3rd and 7th day mixed as presenting transitional characteristic. Furthermore, the last measuring day is clearly separated and presents the lowest PC1 score. However, for the human VH1 and bovine VH4 sample, the discrimination of sample age is not as successful and allows only the 14th measurement day distinction for the VH4 sample and no clear separation between any of the groups for VH1.



**Fig. 4** Comparison of HCA results obtained for the distinction of blank human (VH1, VH2, VH3) and bovine (VH4) vitreous humor samples stored at 4 °C, measured on **A** 1st and **B** 14th day

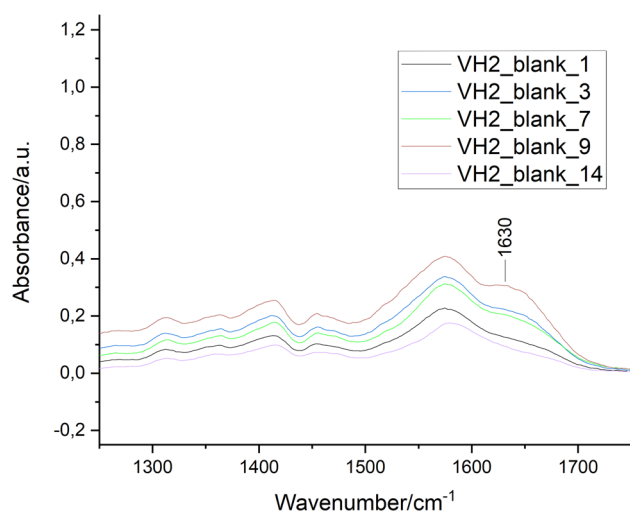
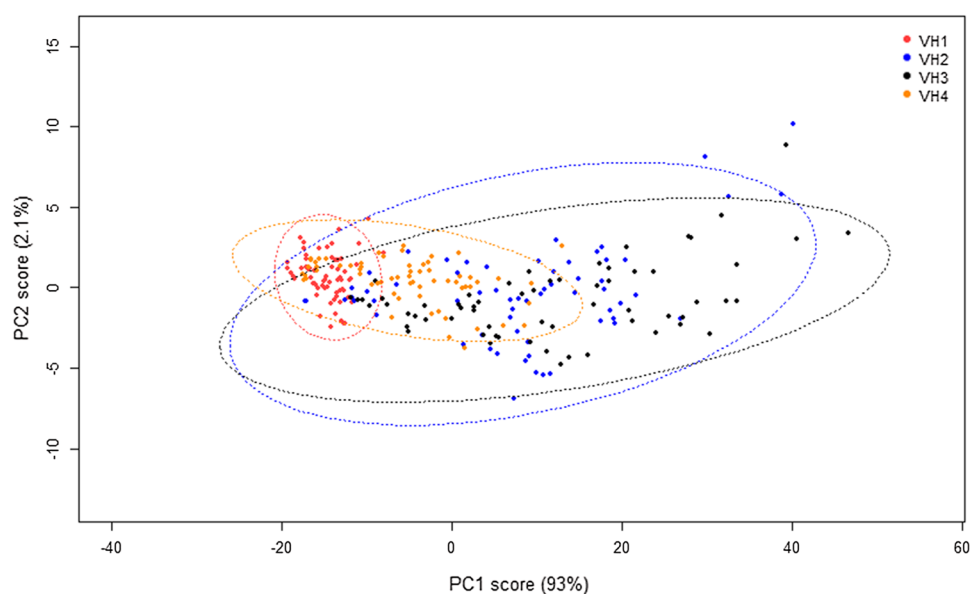


**Fig. 5** Comparison of PCA plots obtained for the distinction of blank human (VH1, VH2, VH3) and bovine (VH4) vitreous humor samples stored at 4 °C, measured on **A** 1st and **B** 14th day



**Fig. 6** Scree plots obtained for PCA analysis of data collected for blank human (VH1, VH2, VH3) and bovine (VH4) vitreous humor samples stored at 4 °C, measured on **A** 1st and **B** 14th day

**Fig. 7** PCA plot obtained for the distinction of human (VH1, VH2, VH3) and bovine (VH4) samples stored at 4 °C, throughout the 14-day study



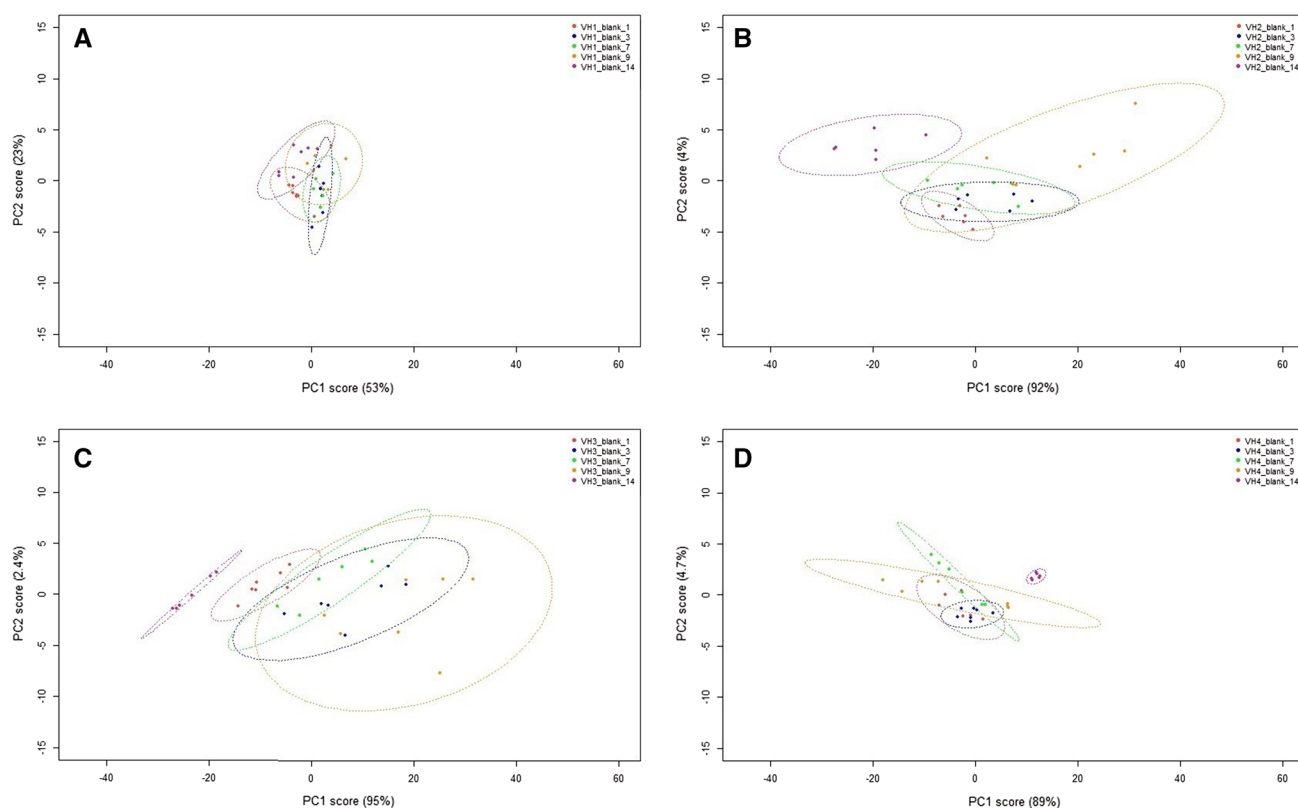
**Fig. 8** Intensity changes of the 1630  $\text{cm}^{-1}$  band for the blank human VH2 sample, stored at 4 °C, measured on 1st, 3rd, 7th, 9th, and 14th days

### Vitreous humor changes under different conditions

VH samples were deproteinized with acetonitrile solutions stored at different conditions: in a freezer ( $-20\text{ }^{\circ}\text{C}$ ) and in ambient laboratory temperature ( $\sim 20\text{ }^{\circ}\text{C}$ ) did not present different FT-IR spectral characteristic. Results obtained for all VH samples studied were undistinguishable after their HCA and PCA analyses. As an example, PCA results obtained for VH2 sample are presented in Fig. 11. Then, it may be concluded that different acetonitrile deproteinization conditions do not affect vitreous humor FT-IR spectra and can be used interchangeably.

Measurement conducted after addition of mephedrone to VH samples did not result in successful drug's detection. There was no significant difference observed in the FT-IR spectra recorded for blank and mephedrone containing samples. Furthermore, HCA and PCA methods were also unsuccessful in the discrimination between blank





**Fig. 9** PCA results obtained for the discrimination of time changes in the spectral characteristic of: **A** VH1, **B** VH2, **C** VH3 (human samples), **D** VH4 (bovine sample), stored in 4 °C, measured on 1st, 3rd, 7th, 9th, and 14th days

and drug-containing samples. As an example, PCA results obtained for VH3 sample are presented in Fig. 12.

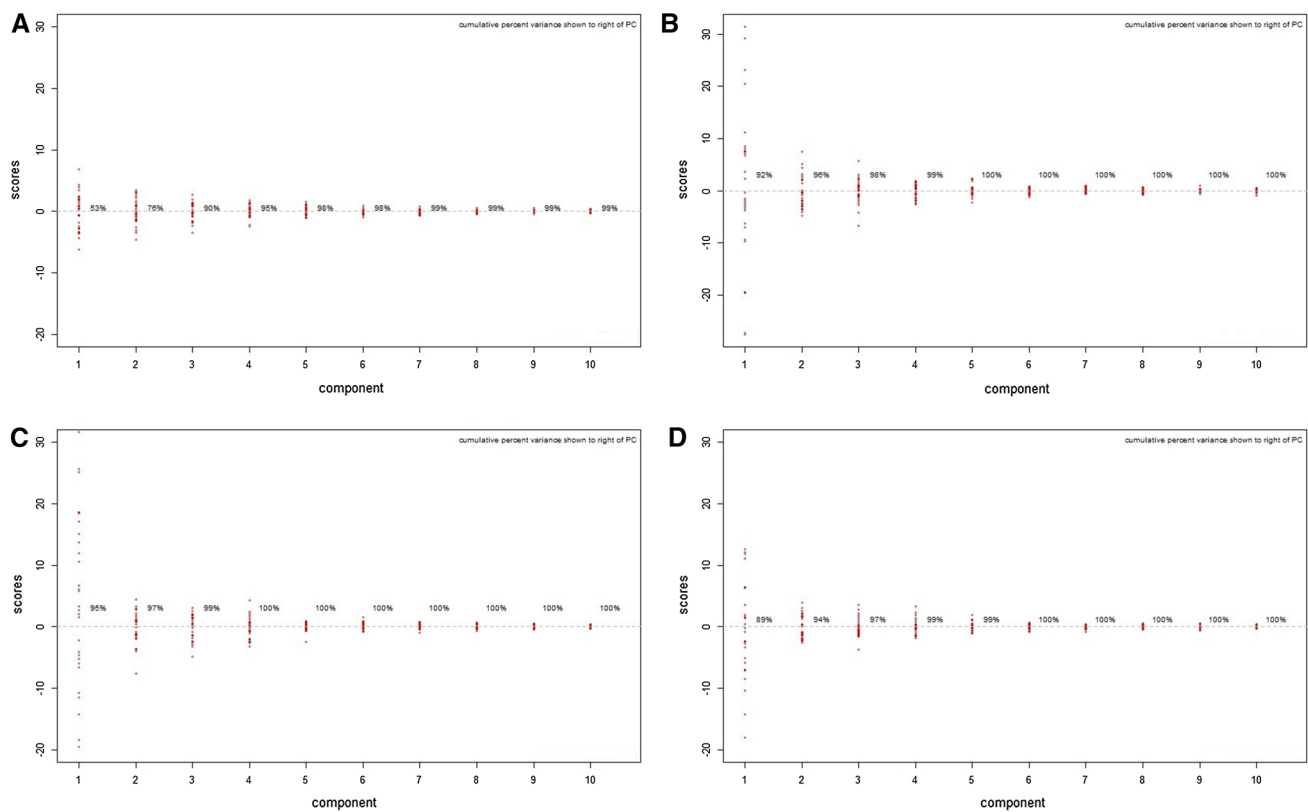
Opportunity to detect drugs in the vitreous humor matrix by simple and non-destructive FT-IR method could be a great advantage for forensic toxicology. However, detection capabilities of FT-IR method are limited and further advances need to be studied to enable detection at such a low concentration level. To check if the detection capability is not diminished by VH matrix, detection of mephedrone in a methanol solution was also examined. 2 mm<sup>3</sup> of mephedrone solution in methanol was deposited on a glass slide, left for 30 min drying and measured by FT-IR. No spectrum was obtained for the concentration of 10 µg/cm<sup>3</sup>, however, the drug's concentration of 1 mg/cm<sup>3</sup> presented successful mephedrone identification as shown in Fig. 13. This concentration is much higher than toxicologically relevant concentrations, therefore, studies regarding addition of mephedrone in concentrations between 10 µg/cm<sup>3</sup> and 1 mg/cm<sup>3</sup> are planned. Capability to detect other psychoactive drugs will be also studied.

## Conclusion

The study proved that the analysis of vitreous humor samples can be performed using the fast and non-destructive FT-IR spectroscopy technique. Visual comparison of FT-IR spectra and further chemometric analyses enabled the discrimination of human and bovine vitreous humor. Differences between human VH samples stored for diverse times (for the long time) were also observed.

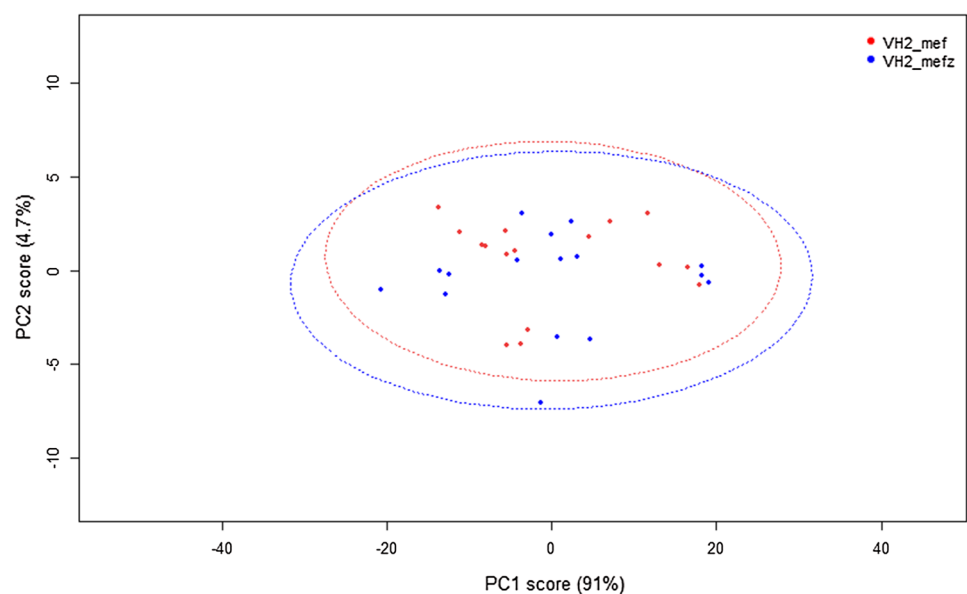
Time changes in vitreous humor samples were identified on FT-IR data collected throughout a 2 weeks' study. PCA analysis enabled the distinction of spectroscopic data. For most of the samples, clear distinction of data obtained for the 14th day was reported. However, changes during the earliest time points were successfully distinguished only for human vitreous humor samples VH2 and VH3. Further studies are required on a larger number of samples including fresh or stored for less than 2 years post-mortem VH human samples. Furthermore, changes during storage times longer than 14 days need to be studied.

FT-IR technique was not able to capture changes related to the mephedrone addition to VH in the concentration of 10 µg/cm<sup>3</sup>. PCA and HCA analyses were unsuccessful in the data discrimination between blank and drug-containing



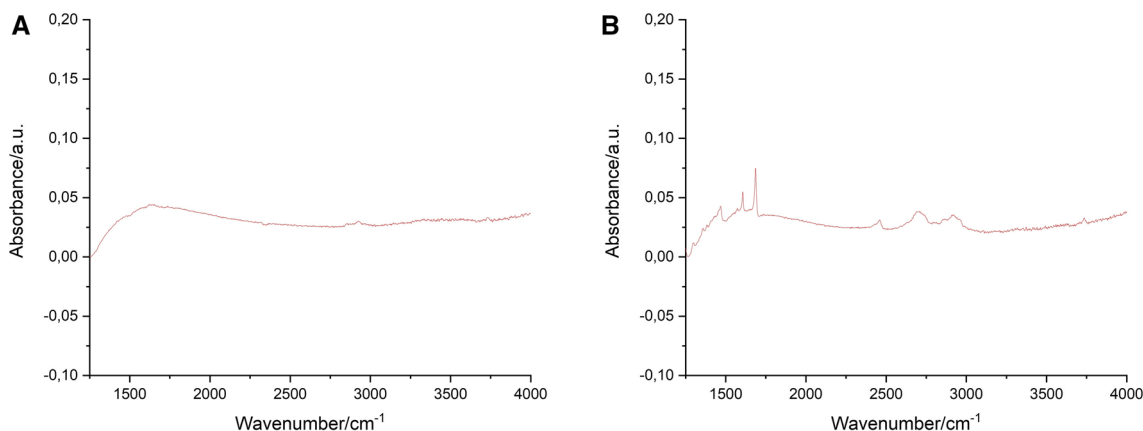
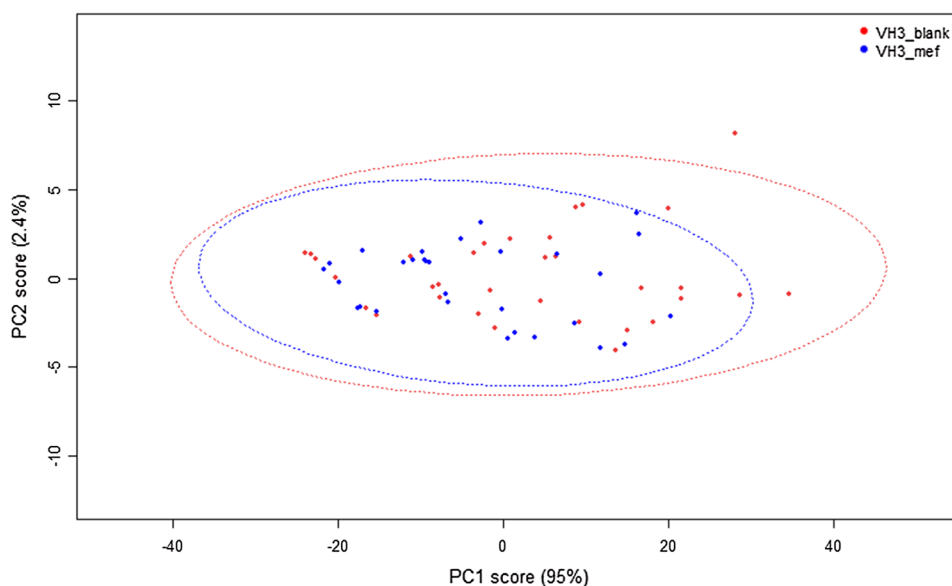
**Fig. 10** Scree plots obtained for PCA analysis of data collected for **A** VH1, **B** VH2, **C** VH3 (human samples), **D** VH4 (bovine sample), stored in 4 °C, measured on 1st, 3rd, 7th, 9th, and 14th days

**Fig. 11** PCA results obtained for the discrimination of human vitreous humor samples containing mephedrone in the concentration of 10 µg/cm<sup>3</sup>, after deproteinization with acetonitrile stored at room temperature (VH2\_mef) and in the freezer at – 20 °C (VH2\_mefz)





**Fig. 12** PCA results obtained for the discrimination of blank human VH samples (VH3\_blank) and mephedrone containing samples (VH3\_mef), stored at 4 °C, measured throughout the 14th-day study



**Fig. 13** FT-IR spectrum of mephedrone standard measured on a glass slide after evaporation of **A** 10  $\mu\text{g}/\text{cm}^3$ , **B** 1  $\text{mg}/\text{cm}^3$  solution in acetonitrile

samples. No time changes that may be associated to drug stability or kinetics were observed. Higher drugs concentrations may be required to obtain their FT-IR detection.

## Experimental

### Sample preparation

Three different human vitreous humor samples collected post-mortem were provided by courtesy of the Institute of Forensic Research and the Forensic Medicine Unit at the Department of Forensic Medicine of the Wrocław Medical University (according to the Bioethical Commission Approval no 1072.6120.303.2018). In addition,

one sample of bovine VH, as a surrogate matrix, was collected from calf eyes supplied by the meat manufacture (Laskopol, Poland). Human VH samples were stored in a freezer ( $-20\text{ }^{\circ}\text{C}$ ) for different times: more than 5 years (VH1) (case Archival X), 3 years (VH2) and 2 years (VH3). Bovine VH sample (VH4) was collected 2 weeks before the analysis and also stored in the freezer.

From each VH sample, two groups were prepared: (1) blank samples containing 200  $\text{mm}^3$  of each VH, and (2) samples containing 200  $\text{mm}^3$  of each VH with mephedrone in a concentration of 10  $\mu\text{g}/\text{cm}^3$ . All samples were left in a fridge ( $4\text{ }^{\circ}\text{C}$ ) to reach equilibrium. After 24 h, samples were centrifuged (14000 rpm, 10 min) and two portions (20  $\text{mm}^3$ ) of each sample's supernatant were collected. 20  $\text{mm}^3$  of acetonitrile stored in a room temperature—first

group of portions, and stored in a freezer—second group of portions, was used for the samples deproteinization. All samples obtained were vortexed for 20 s and centrifuged (14000 rpm, 10 min). 20 mm<sup>3</sup> of each sample's supernatant was subsequently collected, vortexed for 5 s and three drops (2 mm<sup>3</sup>) were deposited on microscope slides, dried for 30 min under fume hood and subjected to FT-IR analysis. The whole procedure was then repeated after 3, 7, 9, and 14 days of sample storage (4 °C).

### FT-IR spectroscopy measurements

ATR FT-IR measurements were performed using Thermo Nicolet iS50 FT-IR (Thermo Fisher Scientific Co, Waltham, MA, USA). Glass slides with samples were placed at appropriate position on a ZnSe crystal and carefully pressed with an ATR pressure tower. The spectra were collected co-adding 32 scans in the range between 550 and 4000 cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup>. After each measurement, the surface of the crystal was cleaned with 50% v/v isopropanol–water solution. A background check was performed before each sample's measurement.

### Statistical data analysis

Spectra processing was performed using Thermo Electron's OMNIC 9 software and it included background correction and normalization by maximum. In addition, the spectra were smoothed using the Savitzky-Golay filter (15 pts) in the R environment (ChemoSpec package). Hierarchical cluster analysis (HCA) and principal component analysis (PCA) was used to provide statistical analysis of the spectra. The spectra were not scaled. Classical PCA option with no scaling was used. For HCA, complete clustering method and Euclidean distance method were applied. Statistical analyses were performed using the R environment equipped with the ChemoSpec package, which is a free software that enables relatively simple and short data analysis.

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